# Molecular Analysis of the Immunoglobulin $V_H$ Gene Rearrangement in a Primary Cutaneous Immunoblastic B-Cell Lymphoma by Micromanipulation and Single-Cell PCR

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**The immunoglobulin $V_H$ gene rearrangement in a primary cutaneous, large-cell (centroblastic and immunoblastic) B-cell lymphoma was analyzed using a micromanipulation/single-cell polymerase chain reaction technique. In all single B cells obtained from CD20-stained skin sections that gave a polymerase chain reaction product (eight of 27 in biopsy I), the same $V_HDJ_H$ rearrangement, consisting of DP-54-DIR1-$J_H$3a genes, was detected, with no intraclonal nucleotide diversity. Comparison with the most closely related germline counterpart showed significantly altered complementarity determining gene regions as a result of somatic mutations, suggesting an antigen-driven selection and expansion of this particular B-cell clone. Interestingly, in a biopsy obtained from the patient 9 mo later, during disease progression (deep muscle infiltration), the lymphoma cells again contained the same $V_HDJ_H$ gene rearrangement (six of 18 in biopsy II) without any further somatic mutations. Therefore, it is suggested that the cutaneous lymphoma characterized throughout this study descended from postgerminal center B-cells.** *Key words: cutaneous B-cell lymphoma/Ig gene rearrangement. J Invest Dermatol 109:541–545, 1997*

Cutaneous B-cell lymphomas (CBCL) are rarely occurring neoplasms characterized by the accumulation of clonally related B lymphocytes in the skin. Like systemic (nodular) lymphomas (Lennert *et al*, 1975), the different types of cutaneous lymphomas (Willemze *et al*, 1996) reflect, to some extent, different stages of the B-cell ontogeny, ranging from immunoblastic lymphomas, to centroblastic-centrocytic (follicle center) lymphomas, to immunocytomas and plasmocytomas. According to the classification recently proposed by the EORTC cutaneous lymphoma study group (Willemze *et al*, 1996), however, cutaneous lymphomas differ from their systemic counterparts by clinical course and therapeutic approaches (Willemze *et al*, 1987; Santucci *et al*, 1991; Slater, 1994).

During their development within the bone marrow, B cells recombine different $V_H$, D, and $J_H$, as well as $V_L$-$J_L$ gene segments to generate multiple templates for a broad heterogeneity of antibody specificities. This fact can be used for the diagnosis of B-cell malignancies, because every distinct B-cell clone is characterized by an individual genomic sequence resulting from unique $V_H$-D-$J_H$, $V_L$-$J_L$ recombination events (Lieber, 1992). Moreover, the nucleotide sequences of the recombined immunoglobulin heavy and light chain encoding gene segments may provide information regarding the stage of B-cell development from which a lymphoma descends. From the pattern of somatic mutations within the $V_H/V_L$ genes one may determine whether the clonal B-cell expansion took place before, during, or after antigen selection (MacLennan and Gray 1986; Berek *et al*, 1991; Küppers *et al*, 1993) resulting in pregerminal center, germinal center, or postgerminal center cell lymphomas, respectively. These studies have systematically been carried out for systemic non-Hodgkin's B-cell lymphomas (Hummel *et al*, 1994). For CBCL such data are not yet available but may provide an important input from the molecular biologic point of view into the further improvement of the classification of such diseases (Rijlaarsdam *et al*, 1996).

The technique of single-cell micromanipulation followed by polymerase chain reaction (PCR) amplification of the particular Ig-gene recombined in this cell is very useful in studying the immunoglobulin genes in B-cell lymphomas (Küppers *et al*, 1994; Küppers *et al*, 1995). Lymphoma cells may be "picked" under visual control from different localizations of a histologic skin section that has been stained with monoclonal antibodies for lineage-specific membrane surface antigens. Using this technique we analysed the clonal immunoglobulin gene $V_H$ rearrangement in a patient with a large-cell centroblastic to immunoblastic cutaneous B-cell lymphoma at different stages during progression.

## MATERIAL AND METHODS

**Patient** A 49-y-old female presented with bluish-red indurated plaques (first appearing in September 1994) and partially ulcerated nodules on the right lower leg. The first biopsy (sample 1) was taken from the right lower leg in December 1994, the second (sample 2) during disease progression in September 1995. A clinical staging carried out in December 1994, as well as 6 mo later, and including x-ray, computer tomography, sonography, blood analysis, and bone marrow biopsy, did not show internal involvement. The patient was treated with a CHOP regimen (six cycles: cyclophosphamide, adriblastine, vincristine, prednisone) resulting in partial remission. As early as 8 wk later the patient presented disease progression and dissemination (lymph node and gastrognemicus muscle involvement) complicated by deep venous thrombosis, dyspnoea, and early renal failure. In October 1995 the whole right leg was treated by electron beam irradiation. Chemotherapy (B-ALL regimen, including methotrexate,

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Abbreviations: CBCL, cutaneous B-cell lymphomas; CDR, complementarity determining region; D, diversity gene segment; FCCL, follicle center cell lymphoma; FR, framework region; $J_H$, joining region gene (heavy chain); $J_L$, joining region gene (light chain); MALT, mucosa-associated lymphoid tissue; PCR, polymerase chain reaction; R:S, ratio of replacement *versus* silent mutations; $V_H$, variable region of the immunoglobulin heavy chain gene; $V_L$, variable region of the immunoglobulin light chain gene.

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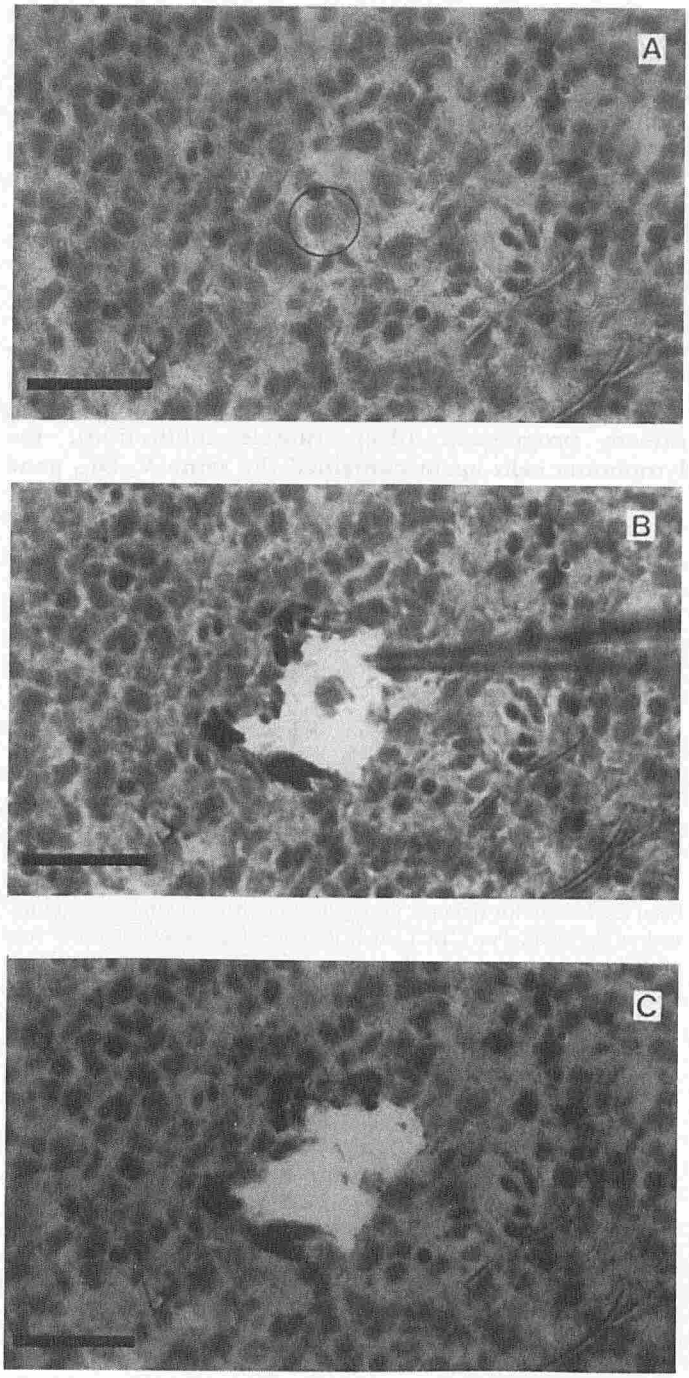


**Figure 1. Micromanipulation to obtain single CD20-stained B cells from a tissue section of a cutaneous B-cell lymphoma.** For details see *Material and Methods*. (*A*) The 10-μm thick cryosection; (*B*) picking a single CD20-positive cell by hydraulic micromanipulation; (*C*) the tissue section after micromanipulation. *Scale bars*, 25 μm.

chlorambucil, doxorubicin, dexamethasone) induced a reduction of intra-abdominal tumor mass but could not stop disease progression. In June 1996 the patient died.

**Histology, immunhistochemistry, and molecular biology** The histology showed the diffuse infiltration of the dermis and subcutis mainly with large immunoblast- and centroblast-like cells that were immunohistochemically characterized as CD20+, CD30-, *bcl*-2+. Most of the cells expressed the lambda light chain isotype. Nearly 100% of the pathologic cell population was in proliferation. In the biopsy obtained in September 1995 (sample 2), deep infiltration by CD20-positive tumor B cells and destruction of the muscular structure became obvious. Routine molecular biologic analysis showed a dominant monoclonal IgH gene rearrangement amplified by $V_H$ framework

**Table I. Oligonucleotide primers for single-cell PCR analysis of rearranged human $V_HDJ_H$ genes[a]**

| Primer | Nucleotide sequence |
|---|---|
| $V_H1$ | 5′ CC TCA GTG AAG GTC(T) TCC TGC AAG GC 3′ |
| $V_H2$ | 5′ GT CCT GCG CTG GTG AAA CCC ACA CA 3′ |
| $V_H3$ | 5′ G GGG TCC CTG AGA CTC TCC TGT GCA G 3′ |
| $V_H4$ | 5′ G ACC CTG TCC CTC ACC TGC G(A)CT GTC 3′ |
| $V_H5$ | 5′ AAA AAG CCC GGG GAG TCT CTG AG(A)G A 3′ |
| $V_H6$ | 5′ ACC TGT GCC ATC TCC GGG GAC AGT G 3′ |
| $J_H1,2,4,5$ extern (3′) | 5′ ACC CTG GTC ACC GTC TCC TCA GGT 3′ |
| $J_H3$ extern (3′) | 5′ ACA ATG GTC ACC GTC TCT TCC GGT A 3′ |
| $J_H6$ extern (3′) | 5′ ACC ACG GTC ACC GTC TCC TCA GGT 3′ |
| $J_H1,4$ intern (5′) | 5′ GAC GGT GAC CAG GGT (T)GCC GCC 3′ |
| $J_H2$ intern (5′) | 5′ GAC AGT GAC CAG GGT GCC ACG GCC 3′ |
| $J_H3$ intern (5′) | 5′ GAC GGT GAC CAT TGT CCC TTG GCC 3′ |
| $J_H6$ intern (5′) | 5′ GAC GGT GAC CGT GGT CCC TTT(G) GCC 3′ |

[a]Primers were designed according to Küppers *et al*, 1994)

3/$J_H$ primers from total DNA obtained from paraffin-embedded skin sections (Willemze *et al*, 1996). The lymphoma therefore was classified as a large B-cell lymphoma (immunoblastic/centroblastic) of the leg (Vermeer *et al*, 1996).

*Obtaining single cells from stained skin sections by micromanipulation* Frozen skin sections (10-μm thick) were stained with the CD20 antibody (Dako, Hamburg, Germany) using biotinylated Fab anti-mouse and streptavidine-alkaline phosphatase complexes (Dako) as developing reagents. Control sections were stained with CD3 antibodies. After washing, bound alkaline phosphatase was visualized by staining with New Fuchsin. The slides were counterstained with hematoxylin.

Single cells were mobilized under the microscope (Nikon, Düsseldorf, Germany) with the help of a hydraulic micromanipulator (Narishige, Düsseldorf, Germany) using 600 × magnification. Then, single cells were aspirated into a micropipette (Narishige), put into 20 μl PCR buffer supplemented by 1 ng rRNA per μl (both from Boehringer, Mannheim, Germany), and stored at −20°C. Photographs were taken before and after the micromanipulation of each cell (**Fig 1*A–C***).

*Molecular biologic analysis of the IgH genes* A set of oligonucleotide primers was used for PCR amplification of rearranged $V_H$ genes (**Table I**). Six $V_H$ gene family specific primers were used together with $J_H$ specific oligonucleotides (Küppers *et al*, 1993). A semi-nested PCR approach was chosen. In the first round of amplification, six $V_H$ gene primers together with the outer (3′) $J_H$ primer mix were used simultaneously in one tube. For the second round of amplification, aliquots of the first round were reamplified using the same $V_H$ primers but nested $J_H$ primer mixes in separate reactions for each $V_H$ gene family. Before PCR, the single cells were incubated with 0.25 mg proteinase K per ml (Boehringer) for 55 min at 50°C and additionally at 95°C for 10 min (enzyme inactivation). The reaction mix (50 μl) for the first round of amplification contained PCR buffer (Boehringer), 2.5 μM $MgCl_2$ (Boehringer), 200 μM of each nucleotide (dATP, dCTG, dGTP, dTTP; AGS, Heidelberg, Germany), 7 nM of each primer, and 3.5 U Expand© High Fidelity Taq-polymerase (Boehringer). PCR amplification was carried out on a Personal Cycler (Biometra, Göttingen, Germany): one cycle at 95°C for 2 min, 65°C for 1 min (when Taq-polymerase was added), and 72°C for 1 min, followed by 35 cycles at 95°C for 30 s, 59°C for 30 s, 72°C for 1 min, and final extension at 72°C for 5 min. The second round of PCR was carried out in separate reactions for each of the six $V_H$ family specific primers using 1 μl of the amplification product from the first round. Reaction mixture: PCR buffer (Perkin-Elmer, Überlingen, Germany), 1.5 μM $MgCl_2$ (Perkin-Elmer), 200 μM of each nucleotide, 7 nM of each primer, and 5U Taq-polymerase (Perkin-Elmer). The second round cycle program was carried out on a TRIO-Thermoblock (BIOMETRA) and consisted of one cycle at 95°C for 2 min, 68°C for 5 min (addition of Taq-polymerase), and 72°C for 1 min, 45 cycles at 95°C for 1 min, 61°C ($V_H1$, $V_H2$, $V_H5$, $V_H6$ specific primers) or 65°C ($V_H3$, $V_H4$) for 30 s, and a final extension at 72°C for 5 min. A 5-μl aliquot of the reaction mixture was analysed on a 2% agarose gel.

Strong attention was paid to avoid contamination with DNA. The micromanipulation, first and second round of PCR were carried out separately in different rooms. As negative controls, two T cells from CD3-stained adjacent sections were picked during the same experiment.

The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) followed by direct sequencing with the Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) on a Sequencing System 373 (Applied Biosystems, Weiterstadt, Germany) using between 0.5 and 10 μl purified PCR product and 1.5 μl 2.5 μM of the family specific $V_H$ primer. Once the used $J_H$ gene became clear the PCR product was

**Table II. Summary of the single-cell analysis of micromanipulated cutaneous lymphoma B cells**

| Experiment (first round PCR) | Biopsy | Cells | PCR products | | Rearrangements |
|---|---|---|---|---|---|
| | | | Total | Sequenced | |
| I | 12/94 | 9 | 3 | 3 | 3 $ILV_H3$ |
| II | 12/94 | 9 | 4 | 4 | 4 $ILV_H3$ |
| III | 12/94 | 9 | 2 | 1 | 1 $ILV_H3$ |
| IV | 9/95 | 9 | 5 | 2 | 2 $ILV_H3a$ |
| V | 9/95 | 9 | 4 | 4 | 4 $ILV_H3a$ |
| Total | | 45 | 18 | 14 | 14 $V_H3$ = ILVH3a |

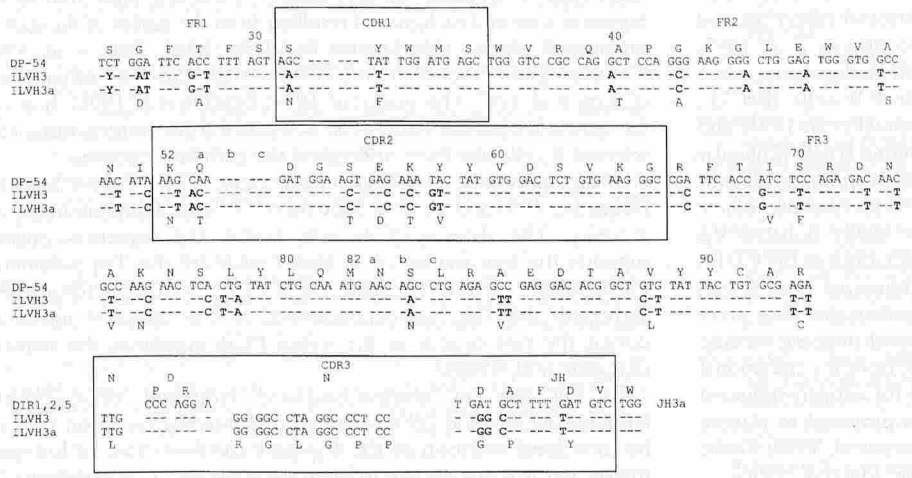

**Figure 2. Nucleotide sequence of the $V_HDJ_H$ rearrangement amplified by PCR from single B cells from tissue sections of a patient with cutaneous B-cell lymphoma.** $ILV_H3$... representative example of $V_H$ genes obtained from biopsy sample 1 (December 1994), $ILV_H3a$... representative sequence obtained from biopsy sample 2 (September 1995). Nucleotide differences in comparison to the most closely related germline gene (DP-54, Tomlinson *et al*, 1992) are indicated by bold letters; dots show nucleotide similarity.

sequenced into the 5′ direction with the corresponding $J_H$ primer. The evaluation was carried out with the help of the software packet "Sequence Navigator" (Applied Biosystems). The sequences were online analysed using the Software "DNA BLOT" (created by H.-H. Althaus) and compared with the gene bank "V BASE Sequence Directory" (Tomlinson *et al*, MRC Centre for Protein Engineering, Cambridge, UK, 1996) for determining the most similar germline $V_H$ genes.

## RESULTS

**Micromanipulation and single-cell PCR data** A total of 45 single CD20-positive B cells were micromanipulated from frozen tissue sections (**Fig 1**) made from two skin biopsies obtained in December 1994 and September 1995, respectively. Five independent first round PCR experiments were carried out on different days (**Table II**). As negative controls, two single T cells per experiment were micromanipulated from a CD3-stained adjacent section of the same biopsy on the same day. From all T cells analysed, no Ig gene rearrangements were obtained by PCR. From a total of 45 CD20-positive B-cells, 14 (31%) yielded amplification products, which after direct sequencing were found to represent identical $V_HDJ_H$ Gene Rearrangements (see below).

**Analysis of $V_HDJ_H$ gene rearrangements in lymphoma B cells** Two representative $V_HDJ_H$-gene sequences amplified from biopsy in December 1994 ($ILV_H3$) and September 1995 ($ILV_H3a$) are shown in **Fig 2**. All 14 PCR products sequenced throughout this study were identical, independent of the disease stage at which biopsies were taken. The $V_H$-gene sequence carried 39 nucleotide differences (18.4%) compared with the most homologous germline gene (DP-54/VH3–07). It may be assumed that most of these nucleotide substitutions result from somatic mutation because the vast majority of $V_H$ genes are known. The somatic mutations were found to be distributed over the complete $V_H$ gene including both framework (FR) and complementarity determining regions (CDR) with a significantly higher number of replacement mutations within the CDRs (**Table III**). Further analysis showed the DP-54 $V_H$ gene to be linked with a segment of seven nucleotides most likely descended from a DIR1, 2, or 5 gene and a $J_H3a$ gene segment. Obviously, at the $V_HD$ and the $DJ_H$ junctions, N-sequences were introduced during recombination, resulting in a CDR3 of 42 nucleotides length (**Fig 2**).

**Table III. Mutations in the $V_h$ gene obtained from single B cells of a patient with cutaneous B-cell lymphoma**

| Gene segment | Mutations | | R:S ratio |
|---|---|---|---|
| | Replacement | Silent | |
| (FR1)[a] | 3 | 2 | 1.5 |
| CDR1 | 2 | 0 | >3 |
| FR2 | 3 | 4 | 0.75 |
| CDR2 | 8 | 0 | >3 |
| FR3 | 9 | 8 | 1.1 |
| Total number of mutations | | | |
| CDR | 10 | 0 | >3 |
| FR | 15 | 14 | 1.1 |

[a](FR1), framework 1 region truncated in the result of primer binding.

## DISCUSSION

In recent years it has become well established that B-cell lymphomas may appear in the skin, presenting localized nodules and plaques composed of a dense nonepidermotropic follicular and/or diffuse infiltrate around vessels and adnexa, without evidence of extracutaneous manifestation at the time of presentation (Burg *et al*, 1984; Sterry and Hauschild, 1989; Santucci *et al*, 1991; Nagatani *et al*, 1993). The large majority of these primary cutaneous lymphomas has been shown to be descended from cells normally found in follicle centers of lymph nodes (small and large cleaved cells or centrocytes; large noncleaved cells or centroblasts) and have therefore been called primary follicle center cell lymphoma (PCFCCL), representing a distinct disease entity with an excellent prognosis (Willemze *et al*, 1987). Both radiotherapy and CHOP polychemotherapy are highly effective modes of treatment for PCFCCL (Rijlaarsdam *et al*, 1996). Primary cutaneous B-cell lymphomas can be distinguished from pseudolymphoma by immuno-

histochemical and molecular biologic analysis of the rearranged immunoglobulin genes (Landa *et al*, 1993; Peris *et al*, 1995).

The diversity of the primary antibody repertoire results from the multiplicity of the V, D, J gene segments for heavy and light chains (combinatorial diversity) as well as from the imprecision of the joining process (junctional diversity). The generation of such a primary repertoire does not require exposure to antigen. A process of somatic hypermutation is induced by the antigenic challenge. The selection of higher affinity antibodies by the antigen results in the maturation of the immune response (Berek *et al*, 1991; Betz *et al*, 1993).

Gene rearrangement and mutational analysis may not only help in the diagnosis of cutaneous lymphomas but also contribute to an improved classification of these distinct entities. Based on the well-established fact that naive pregerminal center B cells carry nonmutated V genes, whereas germinal center B cells and germinal center derived memory B cells express mutated V region genes (Berek *et al*, 1991; Jacob *et al*, 1991; Küppers *et al*, 1993), B-cell malignancies were classified into those descended from pregerminal B cells (B-CLL, mantle cell lymphoma; Meeker *et al*, 1988; Hummel *et al*, 1994) and others descended from germinal or postgerminal center B cells (follicular lymphomas, multiple myeloma, endemic Burkitt's lymphoma; Bakkus *et al*, 1992; Hummel *et al*, 1994; Chapman *et al*, 1995). Nodular follicle center cell lymphomas were shown to express highly mutated $V_H$ genes with a high ratio of replacement to silent mutations in the CDRs in comparison with the FRs (Both *et al*, 1990; Hummel *et al*, 1994), with numerous intraclonal heterogeneities, suggesting that they were descended from germinal center cells in a process of ongoing somatic hypermutation (Cleary *et al*, 1987). Furthermore, from the mutational pattern within the $V_H/V_L$ gene, a potential role for antigen-triggered growth stimulation and clonal expansion may be proposed to play an important role in the genesis of lymphoma (Cleary *et al*, 1987; Garbe *et al*, 1991; Bahler *et al*, 1992; Bessudo *et al*, 1996; Du *et al*, 1996).

The main problem of the molecular biologic analysis of lymphomas is to assure that an amplification product obtained from a cell population (skin section) by PCR represents the lymphoma clone rather than other infiltrating nonmalignant B cells. To study the V gene repertoires, libraries have been constructed by PCR amplification from B-cell populations (see for example, Bessudo *et al*, 1996; Du *et al*, 1996), but it is difficult to exclude the possibility that sequences have been amplified with different efficiencies, resulting in unequal representation of genes in the libraries. Both of these problems may be overcome when analysis is performed on single cells, followed by direct sequencing of the PCR product obtained from individual gene rearrangements (Küppers *et al*, 1995).

This approach was used to analyse single B cells obtained from tissue sections of a patient with a primary cutaneous B-cell lymphoma. A total of 45 CD20-stained cells was analysed by PCR, amplifying the rearranged $V_HDJ_H$ gene segment. PCR products could be obtained in 18 cases, from which 14 were sequenced. Each nucleotide sequence revealed a DP-54 ($V_H3$)-DIR1-$J_H3a$ gene rearrangement containing numerous N-sequences at the $DJ_H$ and $V_HD$ junctions. There were no other $V_HDJ_H$ rearrangements detected, neither in the micromanipulated B cells nor in a total of 10 CD3-positive cells obtained from an adjacent section of the same biopsy on the same day (two per PCR experiment). Only one particular $V_HDJ_H$ rearrangement was detected in B cells of two independent biopsies. This fact makes it more than likely that the clonally expanded malignant lymphoma B-cells were identified.

Surprisingly, there were no other immunoglobulin gene rearrangements detected, which would suggest reactive B cells in the dermal infiltrate. It may only be speculated that the absence of reactive B cells in both biopsies represents a feature of this particular type of lymphoblastic to centroblastic B-cell lymphoma of the lower leg. In a recent study, when analysing the B cells infiltrating plaque lesions in patients with mycosis fungoides, we were able to detect different $V_HDJ_H$ Ig rearrangements suggesting the polyclonal origin of the cells (Förste *et al*, 1997, submitted).

Comparison of the $V_H$ gene rearranged in lymphoma cells with the most closely related germline gene showed numerous mutations distributed over the whole gene segment. The significantly enhanced ratio of replacement to silent mutations (R:S) within the CDR suggests an antigen-driven maturation process of this particular B-cell clone, found to be typical for FCCL (Hummel *et al*, 1994; Bessudo *et al*, 1996; Du *et al*, 1996). Chang and Casali (1995), however, observed that the codons for the CDR of many germline $V_H$ genes are more prone to replacement mutations than those used in most other proteins. Finding higher R:S ratios for the CDR than for the FR segments could therefore result from random nonselected nucleotide base substitutions. Even taking these assumptions into account, the $ILV_H3$ gene rearranged by cutaneous lymphoma B cells in this case still shows deduced R:S ratios in the CDR1/CDR2 that exceed the expected ones from random base substitions. Only some of the mutations appearing within the $V_H$ gene during a certain immune response are responsible for the maturation of antibody affinity; others – including silent mutations – represent a neutral background resulting from the nature of the intrinsic mutational process (MacLennan and Gray 1986; Betz *et al*, 1993). Comparing the data from our studies with those of other groups (Cleary *et al*, 1987; Hummel *et al*, 1994; Bessudo *et al*, 1996), however, we conclude that the lymphoma descended from antigen-stimulated/selected B cells that have undergone the germline reaction.

Analysing a total of 14 sequences, eight of them from biopsy in December 1994 and six from September 1995, we found no intraclonal diversity. The absence of diversity within the sequences obtained supports the low frequency of errors made by the Taq polymerase. Furthermore, the direct sequencing of PCR products is advantageous as regards the Taq polymerase errors, as only mistakes introduced during the first rounds of the initial PCR appear in the sequence (Küppers *et al*, 1995).

In the case of mucosa-associated lymphoid tissue (MALT) lymphomas, Du *et al* (1996) showed that ongoing mutation indicated by intraclonal variation of the $V_H$ gene clearly existed in low-grade tumors but was not evident in high-grade tumor cell populations. The authors suggest that at least the high-grade MALT lymphomas are derived from postgerminal memory B cells and that direct antigenic stimulation may play an important role in the clonal expansion of MALT lymphomas. It is possible that in at least some cases of high-grade lymphomas, the tumor cells partially lost or no longer preserved the normal ability to respond to the activation of the hypermutation mechanism due to acquired additional genetic abnormalities such as p53 inactivation (Du *et al*, 1995) or c-*myc* activation (van Krieken *et al*, 1990). This is in accordance with the observation that high-grade MALT lymphomas, unlike low-grade tumors, lose the growth response to *Helicobacter pylori* T-cell mediated immunologic regulation (Hussell *et al*, 1993).

Taking these assumptions into account, we suggest that this primary cutaneous B-cell lymphoma, classified as a large-cell immunoblastic lymphoma of the lower leg, descended from antigen-selected post germinal center memory B cells. Further studies will show whether this is a general phenomenon for cutaneous B-cell lymphomas of the leg, which have been recently characterized as a distinct entity with an intermediate prognosis (Vermeer *et al*, 1996).

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